AN ABSTRACT OF THE THESIS OF

Tracy L. Shiozawa for the degree of Master of Science in Microbiology presented on September 30, 1999. Title: An Epifluorescence Method for Assessing Viability of Bacteria in Soil Aggregates.

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Peter J. Bottomley

For the past 40 years, fluorescent antibodies have been used to detect and enumerate bacteria in their natural environments. Unfortunately, fluorescent antibodies do not distinguish between live and dead cells because the antibody is usually prepared against the somatic antigens of heat-killed cells. However, immunofluorescence can be used in combination with viability assays to allow the detection of viable cells. The objective of this study was to assess the potential of an immunofluorescence-cell elongation assay to determine the population size, distribution and viability of total soil bacteria and also of a specific indigenous serotype, AR18, of *Rhizobium leguminosarum* bv. trifolii in a Willamette silt loam soil and in different size classes of soil aggregates prepared from the latter (<0.25 and 2-5 mm). A serotype-specific fluorescein-immunoglobulin conjugate was prepared to *R. leguminosarum* bv. trifolii strain AR18 and a general method for separating bacteria from soil was fine-tuned for the Willamette silt loam. *R. leguminosarum* serotype AR18 populations were similar in whole soil (air dried

overnight at room temperature) and in <0.25 and 2-5 mm aggregate size classes prepared from the latter (1.2 to 1.3 x 10^5 cells per gram of soil). However, differences were observed in time of incubation required to detect elongated cells and in the percentages of cells that did elongate from the three soil preparations. For example, after 6 hours incubation, 0, 5 and 8% elongated cells were detected in the 2-5 mm aggregates, <0.25 mm aggregates and whole dried soil, respectively. Population differences were further exaggerated in whole soil and aggregates prepared from soil dried at 4°C for 7 days. Although cells of serotype AR18 elongated in the presence of 30 μg ml^{-1} nalidixic acid, the latter was detrimental to the general bacterial population of the soil. Even after 18 hours incubation, no more than 13.2% of the populations were elongated. However, as with AR18, differences among the 30 μg ml^{-1} nalidixic acid resistant population were detected. The first appearance of elongated cells differed between the <0.25 and the 2-5 mm aggregates prepared from 4°C, 7-day air dried soil (12 versus 18 hours of incubation, respectively). The final percentages of elongated cells were always larger in the <0.25 than in 2-5 mm size classes regardless of how the soil was dried; 5.9 versus 1.4% in aggregates dried at room temperature overnight and 13.2 versus 2.1% in aggregates dried at 4°C for 7 days.

An Epifluorescence Method for Assessing Viability of Bacteria in Soil Aggregates

by

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INTRODUCTION TO THE THESIS

Legumes are used extensively in agriculture both as food and fodder sources for human and domestic animal consumption. Many of the beneficial attributes of leguminous plants can be linked directly to the fact that nitrogen fixing bacteria are capable of forming a symbiosis with legumes, resulting in the reduction of dinitrogen gas to an available form, ammonia. Over the past 100 years there has been considerable interest in studying *Rhizobium* and the factors influencing nodulation, host specificity, nitrogen fixation and the ecology of inoculants and indigenous populations (Fred et al., 1932; Bottomley, 1992). In recent years, considerable interest has been shown in western Oregon and California in the use of legumes as winter cover crops for soils normally used for intensive summer vegetable crop production (Burket et al., 1997). These soils tend to be inherently coarse textured, low in organic matter and are vulnerable to structural deterioration caused by tillage, irrigation and harvest activities (Mendes and Bottomley, 1998). There are a number of advantages to be gained from using legumes as cover crops in these systems that include: recovery of residual fertilizer N before it leaches to groundwater (Brandi-Dohrn et al., 1997), fixation of new N to reduce the requirement of fertilizer N on the summer crop (Ebelhar et al., 1984; Hargrove,

1986), and to increase soil organic matter and improve soil structural properties by incorporation and decomposition of the cover crop residue (Miller and Dick, 1995).

A better understanding is needed of what factors control how soil microorganisms are distributed throughout soil structure, how soil structure influences their activities and turnover and vice versa. Microbial activities are reported to be heterogeneously distributed across soil structural units (aggregates) of different sizes ranging from <0.25 to 5 mm in diameter (Beauchamp and Seech, 1990; Seech and Beauchamp, 1988; Gupta and Germida, 1988; Franzluebbers and Arshad, 1997; Jastrow et al., 1996; Miller and Dick, 1995). However, little is known about the distribution and composition of microbial populations in aggregates.

Fluorescent antibodies (FA) have been used for detection of bacteria in their natural soil habitat and in particular, Gram negative bacterial species (Schmidt et al., 1968; Bohlool and Schmidt, 1970). The high degree of specificity of antibodies to *Rhizobium* and *Bradyrhizobium* serotypes has promoted their extensive use in research (Bohlool and Schmidt, 1970; Bohlool and Schmidt, 1973; Kingsley and Bohlool, 1983; Valdivia et al., 1988; Bottomley and Maggard, 1990; Mendes and Bottomley, 1998). However, antibodies prepared against the heat stable somatic antigens of heat killed cells cannot discriminate between live and dead cells. Bottomley and Maggard (1990) combined the immunofluorescence method with a cell elongation assay (Kogure et al., 1979) to enumerate viable soil populations of serotypes of *Rhizobium leguminosarum* bv. trifolii. The cell

elongation assay is based on the use of the DNA gyrase inhibitor, nalidixic acid, which permits transcription and translation but prevents DNA replication. This action inhibits cell division while allowing biomass to increase resulting in the formation of elongated cells ($\geq$ 4.2 μm) which can then be detected by microscopic examination.

In an earlier study (1996-1998), a particular serotype, AR18, of *R. leguminosarum* bv. trifolii was detected in a high percentage of root nodules found on a red clover cover crop growing in soil at the N. Willamette Research and Extension Center, Aurora, OR. Furthermore, the population was detected in a variety of soil aggregate size classes prepared from this soil (Mendes and Bottomley, 1998). This observation prompted me to consider an examination of the ecology of this representative of *R. leguminosarum* bv. trifolii in the different aggregate niches. An obvious question that arose related to the viability of the populations in the different aggregate size classes.

The objectives of this study were: (i) to develop a fluorescent antibody to *Rhizobium leguminosarum* bv. trifolii serotype AR18, (ii) to optimize the viability assay in Willamette silt loam soil and (iii) to examine population size, distribution and viability of total soil bacteria and *R. leguminosarum* bv. trifolii serotype AR18 in two aggregate sizes, <0.25 and 2-5 mm. Because aggregates can be prepared from air dried soil, I had an opportunity to examine the protection offered by these aggregates to desiccation stress.

MATERIALS AND METHODS

Experimental site and soil sampling

Soil samples were collected from the North Willamette Research and Extension Center, Aurora, Oregon. The soil is a Willamette silt loam, Pachic Ultic Argixeroll. The field is managed with three winter rotating crops and two alternating summer crops. The summer vegetable crops are sweet corn (*Zea mays* L. cv. Jubilee) and broccoli (*Brassica oleracea* L. botrytis group cv. Gem) (Mendes and Bottomley, 1998). The winter crop treatments are: winter fallow, red clover (*Trifolium pratense* L. var. Kenland) and triticale (*X Triticosecale wittmack*) (Mendes and Bottomley, 1998). Soil samples were taken from the plots that were planted to red clover and specifically, the subplots which received an intermediate level of N fertilizer (56 kg N ha^{-1}) during the summer crop of sweet corn. The goal of the sampling was to recover soil from plots in which the red clover had grown vigorously and, presumably, would contain a substantial population of *Rhizobium leguminosarum* bv. trifolii. Soil samples were collected in the first week of September, 1998, with a shovel to a depth of 10 cm. Four samples were taken from each replicate plot, mixed in a plastic basin by hand and placed in large reclosable plastic bags for transport to the laboratory.

Soil sieving and aggregates distribution

A composite sample of soil was prepared by mixing equal amounts (250 g) of soil recovered from the four replicate plots. Soil was sieved through a 5-mm screen and incubated in capped mason jars at field moisture content at 25°C for 10 days. This was followed either by drying the soil at 4°C for 7 days, or by drying at room temperature overnight. The field moisture of the soil at the time of sampling was 21% and was reduced by drying at room temperature overnight to 3.1, 2.6 and 3.4% for whole soil, <0.25 and 2-5 mm aggregate size classes respectively. By drying at 4°C for 7 days, soil moisture of the <0.25 and 2-5 mm aggregates was reduced to 3.9 and 5.7%, respectively. Soil was prepared as described above and stored at 4°C in a reclosable plastic bag.

Aggregates were prepared from the dried soil by placing 100 g amounts into the top sieve of a nested stack in a Tyler Ro-Tap sieve shaker (Combustion Engineering Inc., Mentor, OH). Soil was sieved into the following aggregate size classes: <0.25, 0.25 to 0.50, 0.50 to 1.00, 1.00 to 2.00 and 2.00 to 5.00 mm. Sieving periods lasted 3 minutes and aggregates were collected in plastic reclosable bags and stored at 4°C. The preparations of soil associated with each aggregate-size class are: <0.25 (15.7%), 0.25 to 0.50 (11.0%), 0.50 to 1.00 (23.6%), 1.00 to 2.00 (28.4%) and 2.00 to 5.00 (21.3%).

Preparation of rhodamine-gelatin conjugate

Rhodamine-gelatin conjugate used to suppress non-specific fluorescence during immunofluorescence staining was prepared by following a published procedure (Bohlool and Schmidt, 1968). 2 g Difco Bacto-gelatin (Difco Laboratories, Detroit, MI) was dissolved in 100 ml distilled water in a 250-ml Erlenmeyer flask. The flask was warmed gently to promote dissolution of gelatin and then adjusted to a pH of 10.5 using 1 M KOH. The solution was autoclaved for 10 minutes and immediately placed into a cold water bath. The pH was re-adjusted to 10.5 as described previously. To a minimum amount of acetone (approximately 1 ml), 4 mg Rhodamine B Isothiocyanate (Sigma Co., St. Louis, MO) was added to a final concentration of 8 mg g^{-1} gelatin, followed by 25 ml of gelatin solution and stirred overnight in the dark with a 1 cm stir bar. The rhodamine-gelatin conjugate was separated from the non-conjugated dye on a column packed with swollen Sephadex G-25 coarse beads (Pharmacia Fine Chemicals, Piscataway, NJ). The mobile phase was collected using a Gilson Micro-Fractionator (Gilson Medical Electronics Inc., Middleton, WI) and the anti-microbial agent, thimerosal, was added to a final concentration of 0.01%. The rhodamine-gelatin conjugate was dispensed into 3 ml aliquots in scintillation vials and stored at -20°C. Prior to use, rhodamine-gelatin was diluted 1:2 in 0.02 M phosphate buffered saline (PBS), pH 7.2, which promoted uniform spreading of the solution over the surface of Osmonics blackened polycarbonate filters (Osmonics, Livermore, CA).

Preparation of fluorescein conjugated antibody to *Rhizobium leguminosarum* bv. trifolii serotype AR18

R. leguminosarum bv. trifolii serotype AR18 was detected using a fluorescent antibody prepared by conjugating fluorescein isothiocyanate, FITC, (Sigma Co., St. Louis, MO) directly to immunoglobulin (IgG) (Schmidt et al., 1968). Immunoglobulin was separated from whole antiserum as follows. 10 ml ice cold 3.9 M $(NH_4)_2SO_4$ was added dropwise to 10 ml undiluted antiserum (agglutinating titer 1:640) with continuous stirring. The suspension was allowed to stand for one hour at 4°C followed by centrifugation (10,000 x g, 30 min) to separate the precipitated immunoglobulins. Supernatant was decanted and precipitate dissolved in 10 ml of 0.85% w/v saline. The procedure was repeated once to obtain a white precipitate free of hemoglobin. The precipitate was dissolved in 2 ml water and dialyzed against carbonate/bicarbonate buffer, pH 9.0 until the dialysate appeared clear. The protein concentration was determined spectrophotometrically by measuring absorbance at 280 nm using a Pharmacia Biotech GeneQuant II (Pharmacia Biotech, Cambridge, England). The sample was adjusted to 1.0% protein (10 mg ml^{-1}) with 0.85% w/v saline. Four ml of 0.1 M Na_2HPO_4 (pH 9.0) were added to 10 ml of the 1.0% w/v globulin solution followed by 4 ml of 0.1 M Na_2HPO_4 (pH 8.0) containing FITC-10% celite to a final concentration of 0.05 mg FITC per mg of protein. The pH was adjusted to 9.5 with 0.1 N NaOH and adjusted to 20 ml volume with 0.85% w/v saline. Thimerosal (final concentration 0.01% w/v) was added and conjugation allowed to proceed for

20 minutes in a 40°C water bath in subdued light with occasional stirring. The conjugate was divided into aliquots and centrifuged (12,500 x g, 3 min), recombined, and applied to a Sephadex column of coarse G-25 beads. The preparation was eluted with 0.02 M PBS and 2 ml fractions were collected as described above.

Fluorescein:protein ratios were determined for each fraction by measuring optical densities (OD) at 495 and 280 nm and the formula: 2.87 x OD_{495} /OD_{280} - (0.35 x OD_{495}). Those fractions with the highest values were tested for specificity to *R. leguminosarum* bv. trifolii serotype AR18 by their reactivity to nine strains of *R. leguminosarum* representing antigenically distinct serotypes. Results are listed in Table 1. Smears were prepared from broth cultures grown for 48 hours in Yeast Extract Mannitol (YEM) at 25°C with shaking (250 rotations per minute in the Controlled Environment Incubator Shaker, New Brunswick Scientific Co., Edison, NJ). Optical densities of the cultures were measured at wavelength 600 nm and adjusted to an OD of 0.1 or approximately 1.0 x 10^8 cells ml^{-1}. Ten µl of cell suspensions were spread on microscope slides. Smears were air dried, heat fixed and incubated with 1:10, 1:25, 1:50 or 1:100 fluorescent antibody dilutions prepared in 0.02 M PBS, pH 7.2. After 30 minutes, smears were rinsed with 0.02 M PBS, pH 7.2, blotted dry and examined under a Zeiss epifluorescent microscope (Carl Zeiss Inc., New York) as described elsewhere (Demezas, 1986). Total counts and elongated bacterial cells were determined with the aid of a calibrated whipple disk inserted into the eyepiece of the microscope. Cells fluoresced bright green and

were recorded on an arbitrary scale of 0 to 4+ with 4+ being the brightest and 0 being no fluorescence.

Determination of viability of *R. leguminosarum* bv. trifolii serotype AR18 by immunofluorescence and cell elongation

The viability of *R. leguminosarum* bv. trifolii serotype AR18 was evaluated using the immunofluorescence cell elongation assay (Bottomley and Maggard, 1990). Because the cell elongation method had not been tested previously with Willamette silt loam, preliminary experiments were needed to validate various aspects of the experimental procedures.

Ability of *R. leguminosarum* bv. trifolii serotype AR18 to pass through the sequential filtration steps

8, 5 and 3 μm pore-size Osmonics filters were used to separate bacteria from the majority of the sand, silt and debris in the soil which could interfere with staining and microscopic detection of the bacteria. In the absence of soil, four flasks containing 200 ml distilled water were spiked with 1.0×10^7 *R. leguminosarum* bv. trifolii serotype AR18 cells and the suspension passed sequentially through a series of different combinations of filters: 8, 5, 3 or 8, 5 or 5, 3. For each of three replicate filters, 10 ml portions of the suspensions were filtered onto 0.45 μm pore-size blackened filters and the cells enumerated by

immunofluorescence. These values were compared with controls filtered directly onto 0.45 μm pore-size filters.

Length of settling time after soil dispersion

3 g of soil were spiked with approximately 1.0×10^7 cells g^{-1} *R. leguminosarum* bv. trifolii serotype AR18 and mechanically dispersed in 160 ml milk bottles containing 27 ml of 0.15 M NaCl and 12 g of 5 mm glass beads for 10 minutes. Following dispersion, the soil slurry was allowed to settle either 2.5, 5.0 or 10.0 minutes. 10 ml volumes of supernatant were removed after the allotted times, added to 163 ml distilled water and filtered sequentially through 8, 5 and 3 μm pore-size filters. For each of three replicate filters, 10 ml of supernatant were filtered onto a 0.45 μm pore-size blackened filter and cells enumerated by immunofluorescence.

Determining the efficiency of recovery of *R. leguminosarum* bv. trifolii serotype AR18

3 g of soil were spiked with 1.0×10^7 cells g^{-1} *R. leguminosarum* bv. trifolii serotype AR18 and mechanically dispersed as described above. Following dispersion, the soil was allowed to settle for 5 minutes and 10 ml supernatant was removed and added to a 163 ml volume of deionized water. This suspension was filtered through 8, 5 and 3 μm filters and then three replicate 10 ml volumes were

filtered onto 0.45 μm pore-size filters and enumerated by immunofluorescence. A comparison was made with the same number of cells added to 27 ml of 0.15 M NaCl and taken through the same procedure.

The final procedure used for the immunofluorescence-viability assay of *R. leguminosarum* bv. trifolii serotype AR18

Three g portions of soil were suspended in 27.0 ml of 0.15 M NaCl in 160 ml milk bottles with 12 g portions of 5 mm glass beads. Soil suspensions were shaken on a wrist action shaker for 10 minutes, removed, and allowed to settle for exactly five minutes. Ten ml of the supernatant was removed and added to 163 ml distilled water, which was filtered consecutively through 8, 5 and 3 μm pore-size filters. To the filtered soil solution, 20 ml of mineral salts solution of the following composition was added: 2 g $MgSO_4$, 0.8 g $CaCl_2$, 0.5 g K_2HPO_4 and 1.7 g K_2SO_4. 7 ml yeast extract was added (12 g L^{-1} stock solution, 0.2 μm filter sterilized) to a final concentration of 0.4 g L^{-1}. Aliquots of 35 ml were dispensed into stoppered 250-ml Erlenmeyer flasks and nalidixic acid (1.5 mg mL^{-1} in 0.01 M NaOH) was added to either 10, 20 or 30 μg final concentrations (final pH was not changed significantly by the addition of nalidixic acid, pH 7.4, 7.5 and 7.5, respectively). At time intervals of 0, 6, 12 and 18 hours, replicate flasks were sacrificed by addition of formalin (2% v/v, final concentration) and immediately placed at 4°C until processed for microscopy (Bottomley, 1994).

In dim light, fluorescent staining was accomplished by the addition of two drops of the fluorescent antibody (diluted 1:25 in 0.02 M PBS, pH 7.2) to the filter and placed in a humidified chamber for 30 minutes. The membranes were transferred back to the filter supports and destained with 50 ml 0.02 M PBS, pH 7.2, under -50 kPa suction pressure and remounted on microscope slides. To prevent fading of the fluorescence, one drop of Difco FA mounting fluid, pH 7.2 (Difco Laboratories, Detroit, MI), was placed on the top end of the filter which was covered with a 22 x 60 mm coverslip and followed by one drop of Type B immersion oil (R. P. Cargille Laboratories, Inc., Cedar Grove, NJ). 25 fields of view were counted per slide. Viable cells were considered those that had elongated $\geq$ 4.2 μm in length. *R. leguminosarum* bv. trifolii serotype AR18 cells were typically 2 μm in length prior to elongation.

Determination of total bacterial cell counts by epifluorescence microscopy after staining with acridine orange

The total bacterial cell counts were determined using acridine orange (Sigma Co., St. Louis, MO) as essentially described elsewhere (Meyer-Reil, 1978). Samples (1.9 ml) of the formalin-fixed preparations were mixed with 0.1 ml acridine orange (0.1% w/v in 0.1 M citrate buffer, pH 6.6, 0.2 μm filter sterilized) and incubated in subdued light for 10 minutes at room temperature. Uniform dispersion of the cells upon the blackened polycarbonate filters was facilitated by adding an aliquot (0.5 ml) of the acridine orange stained suspension to 10 ml

distilled water. While slight suction remained on the filters, 20 ml portions of 0.1 M citrate buffers, pH 6.6, pH 5.5 and pH 4.0, followed by 20 ml distilled water were sequentially drawn through each filter. Each filter was placed directly on top of a drop of Type B immersion oil on the microscope slides. One drop of Difco FA mounting fluid was added and a coverslip was added to the slide before viewing. Total counts and elongated cell numbers were determined as described above. Cells fluoresced bright green or red. On occasion, photographs were taken of the specimens with a Contax RTS II Quartz camera using Kodak Elite Chrome color slide 100 film and an exposure time of 16 seconds.

RESULTS

Fluorescent antibody conjugate specificity

Although the antiserum to *Rhizobium leguminosarum* bv. trifolii strain AR18 was produced about 10 years ago, a fluorescein-immunoglobulin conjugate was not available for this work. A conjugate was successfully prepared with standard procedures and eight fractions with fluorescein to protein ratios of 1:2 to 1:4 were obtained. Initially, the eight fractions were tested for reactivity against the parent strain AR18 and an antigenically unrelated *R. leguminosarum* bv. trifolii strain, AS36. Based on these results, fractions 3 and 4 were further tested for reactivity against seven additional antigenically distinct *R. leguminosarum* bv. trifolii strains. Fraction 4 (titer 1:25) gave no strong positive reactions except to the parent strain (Table 1). The green fluorescence of the parent strain is illustrated in Fig. 1.

Effect of filtration through 8, 5 and 3 μm pore-size filters

Previous work with different soils had shown that sequential filtration of 1:200 dilutions of soil suspensions through filters of decreasing pore size was a simple procedure for separating bacteria from soil particles which interfere with staining and detection. When cells of *R. leguminosarum* bv. trifolii strain AR18 were passed sequentially through various combinations of 8, 5 and 3 μm pore-size

Table 1. The nine strains tested for reactivity against fluorescent antibody fraction 4, 1:25 dilution.

Strains:	Reaction:
AR18, parent strain	4+
AS36	0
AR16	0
AR21	0
AR23	0
AS6	2+
AS21	0
AP17	0
Pra(0)6	0

*The strength of reaction is noted on a scale from 4+ to 0, with 4+ being the brightest fluorescing cells and 0 being undetectable.

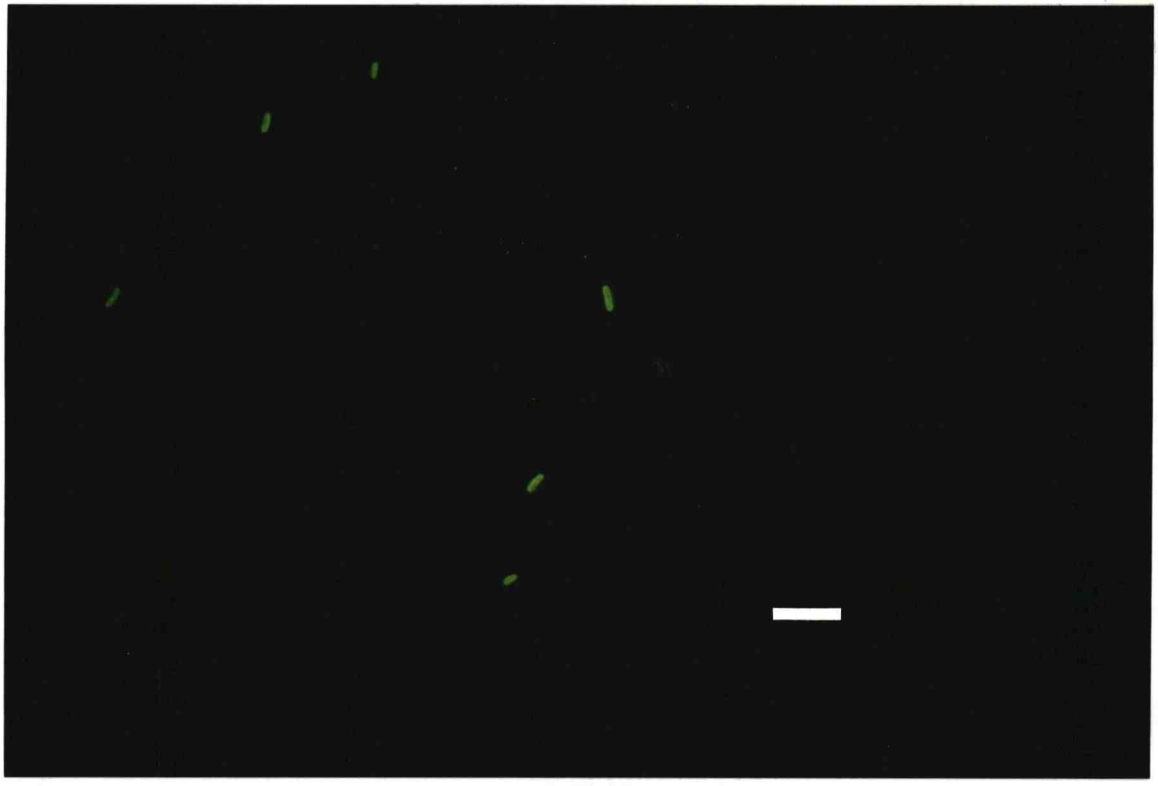

Figure 1. *Rhizobium leguminosarum* bv. trifolii strain AR18 detected using fluorescent antibody, fraction 4 diluted 1:25. The length of the calibration bar is equal to 10 μm. (Magnified 1000X).

filters, combinations 8, 5 and 3 μm, and 8 and 5 μm passed 70-75% of the cells of strain AR18 while the combination of 5 and 3 μm pore-size filters passed all of the AR18 cells (Fig. 2). Although the data indicate that the 8 μm pore-size filter should not be used, I decided to continue to use it because of its importance in removing soil debris from suspensions of cells in soil.

Soil settling time

Following mechanical dispersion, the 1:10 dilution of soil slurry was allowed to sit undisturbed prior to making the 1:200 dilution so that a large part of the dispersed soil might settle out of the suspension. Settling times of 2.5, 5 or 10 minutes were used to determine if settling time influenced the number of cells recovered. Results indicated that while 5.0 minutes of settling yielded the highest average number of cells per field of view, the results of the three times of settling were not significantly different from each other (data not shown). 5.0 minutes settling time was selected for use in this study.

Efficiency of recovery

Incomplete separation of rhizobia from soil, soil settling, and filtration combine to reduce the efficiency with which bacteria are recovered from soil. A comparison made between soil spiked with strain AR18 and a soil-less suspension of strain AR18 indicated a recovery efficiency of approximately 63% (Fig. 3). As a

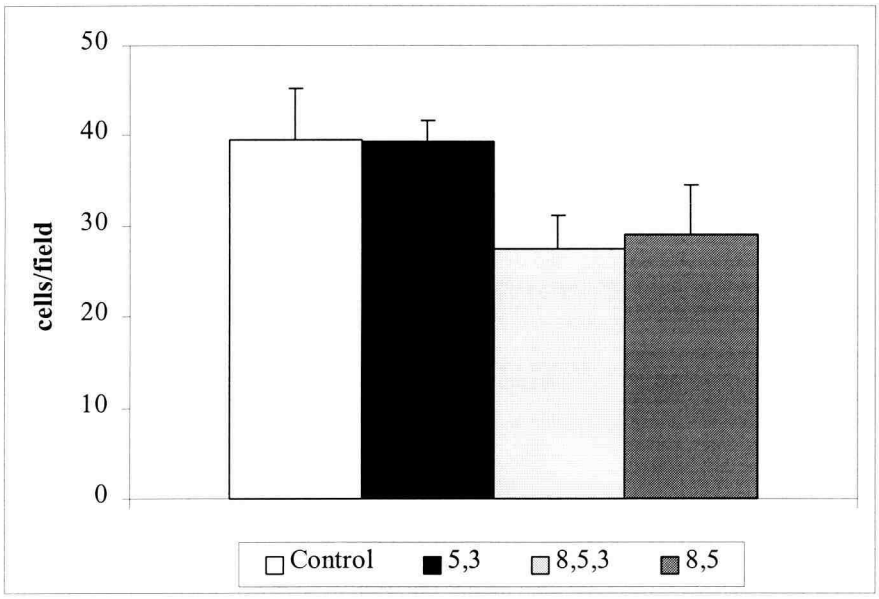

Figure 2. Number of *Rhizobium leguminosarum* bv. trifolii strain AR18 detected with immunofluorescence following filtration through different combinations of filters of different pore sizes (μm). The control represents cells that were filtered directly onto a 0.45μm pore-size filter. Error bars represent the standard deviation of the mean of three replicates.

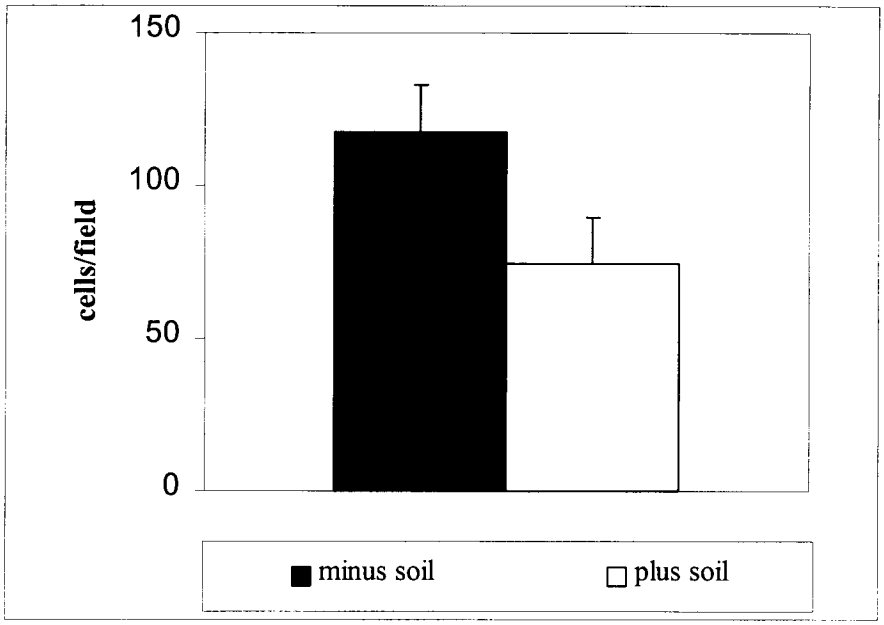

Figure 3. Efficiency of recovery determined by filtering a soil slurry spiked with *R. leguminosarum* bv. trifolii strain AR18 through a combination of 8, 5 and 3 μm pore-size filters. The minus soil treatment represents a suspension of cells filtered directly onto a 0.45 μm pore-size blackened filter for staining. Error bars represent the standard deviation of the mean of three replicates.

consequence, observed immunofluorescence and total bacterial counts were corrected with an efficiency recovery factor of 1.6.

Evaluation of nalidixic acid as a cell division inhibitor for *R. leguminosarum* bv. trifolii strain AR18

Nalidixic acid (Nal) is a DNA gyrase inhibitor which prevents cell division from occurring while allowing transcription and translation to proceed in response to substrate presence. The outcome of exposure to Nal and substrate is cell elongation. Preliminary studies with *R. leguminosarum* bv. trifolii strain AR18 showed that 10 and 20 $\mu g\ ml^{-1}$ Nal could not prevent cell multiplication over 18 hours. 30 $\mu g\ ml^{-1}$ Nal however, resulted in 100% elongation of cells after 18 hours of exposure. These effects were confirmed upon examination of soil borne populations of serotype AR18. Lower concentrations of nalidixic acid, 10 and 20 $\mu g\ ml^{-1}$, could not prevent multiplication of the antigenically related bacteria and lower percentages of elongated cells were observed (data not shown).

Detection and enumeration of *Rhizobium* serotype AR18 from moist and air dried soil

Populations of bacteria antigenically related to serotype AR18 were detected in samples of both moist and air dried soil samples at densities of approximately 10^5 cells g^{-1} soil (Fig. 4). The population was twice as large in moist as in air dried soil (Table 2). Furthermore, almost identical population densities of

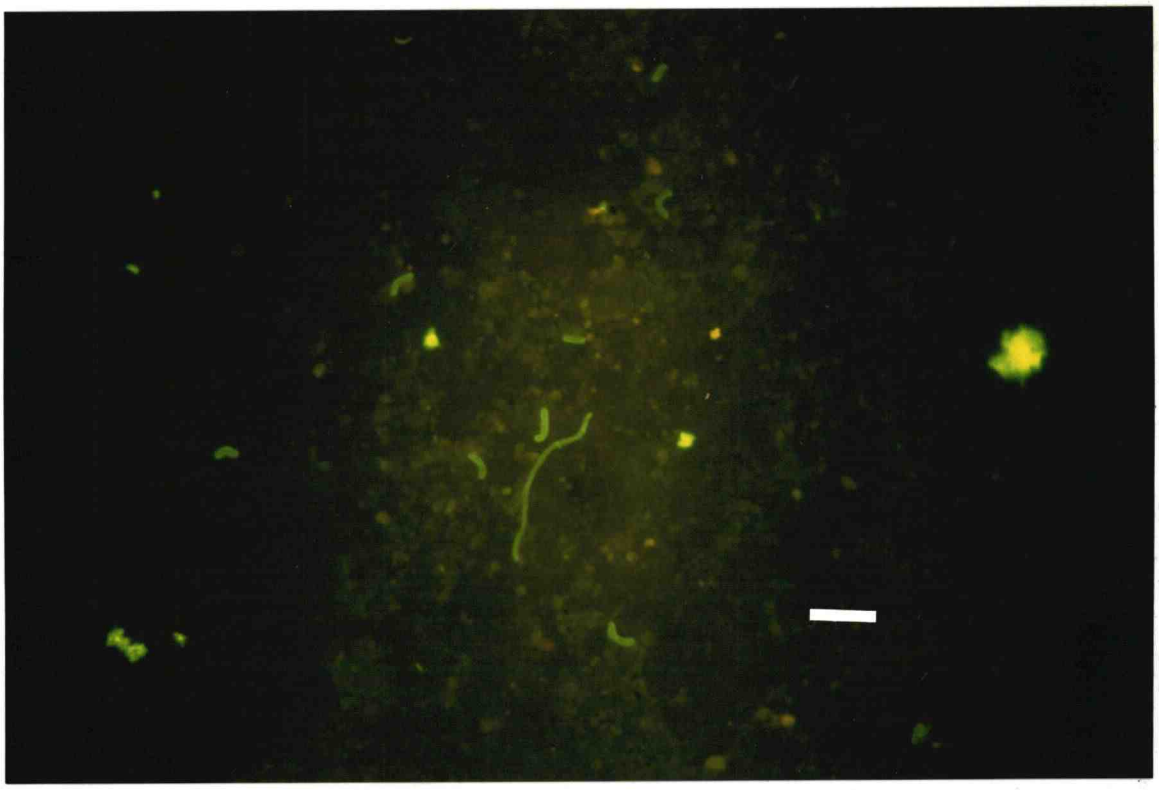

Figure 4. After 6 hours incubation, indigenous *Rhizobium leguminosarum* bv. trifolii serotype AR18 detected using fluorescent antibody, fraction 4, diluted 1:25. The length of the calibration bar is equal to 10 μm. (Magnified 1000X).

Table 2. Population densities of *Rhizobium leguminosarum* bv. trifolii serotype AR18 detected during an 18-hour incubation with yeast extract (0.4 g L^{-1}) and nalidixic acid (30 mg L^{-1}).

	No. of cells g^{-1} soil x 10^{-4} after incubation for (h)			
Soil samples	0	6	12	18
moist soil	32.7	38.5	37.0	186.7
dried soil	13.3	16.7	23.0	115.0
<0.25 mm, o.n.	12.0	24.4	22.0	126.0
2-5 mm, o.n.	13.4	11.2	17.1	56.0
<0.25 mm, 7 days	11.0	11.0	19.0	111.0
2-5 mm, 7 days	8.0	6.4	8.9	27.0

†Numbers in parentheses represent the standard deviation of the mean of three replicates.
‡Whole moist soil contained 21% water.
[1]Soil dried at room temperature, overnight. Whole dried soil contained 3.1% water.
[2]Aggregates prepared from whole soil dried at room temperature, overnight. <0.25 mm aggregates contained 2.6% water and 2-5 mm aggregates contained 3.4% water.
[3]Aggregates prepared from whole soil dried at 4°C, 7 days. <0.25 mm aggregates contained 3.9% water and 2-5 mm aggregates contained 5.7% water.

serotype AR18 were detected in aggregates size classes <0.25 and 2-5 mm. Although 30 μg ml^{-1} Nal was adequate to control proliferation of strain AR18, this was not completely true of bacteria antigenically related to serotype AR18 in the soil. Between 12 and 18 hours, the population of serotype AR18 increased about 5-fold in suspensions from whole soil and <0.25 mm aggregates. In the case of 2-5 mm aggregates, the increase was less (3-fold). Despite the similar population sizes in the aggregates, the <0.25 mm serotype AR18 population from the overnight dried soil responded differently to the presence of yeast extract and nalidixic acid than did the population in the 2-5 mm size class. In the <0.25 mm aggregates, a small number of cells (5%) had elongated after 6 hours, while none were detected in the population from the 2-5 mm aggregates (Table 3). After 12 hours of incubation only 7% had elongated in the 2-5 mm size class, while 15.6% of cells had elongated from the <0.25 mm size class (Table 3). By 18 hours, approximately 50% of the cells detected were elongated in both aggregate fractions. It should be noted that the AR18 population recovered from the whole dried soil responded to YE and Nal more similarly to the <0.25 mm population than to the 2-5 mm population.

The response of the two aggregate size-class populations of serotype AR18 were examined in soil that was dried at 4°C for 7 days prior to aggregate preparation. Although the <0.25 and 2-5 mm size classes contained similar AR18 populations as the other soil samples, there was a greater difference in responsiveness of the <0.25 mm population relative to the 2-5 mm size class (Table

Table 3. Percentage of elongated cells of *Rhizobium leguminosarum* bv. trifolii serotype AR18 detected during an 18-hour incubation.

	Time of Incubation (hours)			
Soil samples	0	6	12	18
Whole moist soil	0 (± 0)	5.2 (± 1.3)	18.7 (± 5.6)	49.7 (± 5.7)
Whole dried soil[1]	0 (± 0)	7.9 (± 11.2)	32.7 (± 11.0)	64.0 (± 8.3)
<0.25 mm aggregates[2]	0 (± 0)	5.2 (± 0.8)	15.6 (± 7.3)	53.4 (± 7.4)
2-5 mm aggregates[2]	0 (± 0)	0 (± 0)	7.4 (± 0.9)	47.3 (± 3.7)
<0.25 mm aggregates[3]	0 (± 0)	12.1 (± 14.8)	30.2 (± 2.8)	54.0 (± 3.7)
2-5 mm aggregates[3]	0 (± 0)	0 (± 0)	4.2 (± 7.2)	33.0 (± 14.7)

[†]Numbers in parentheses represent the standard deviation of the mean of three replicates.
[1]Soil dried at room temperature, overnight.
[2]Aggregates prepared from soil dried at room temperature, overnight.
[3]Aggregates prepared from soil dried at 4°C, 7 days.

2). Approximately 30% of the <0.25 aggregate size class population had elongated after 12 hours incubation in contrast to 4% of the AR18 population present in the 2-5 mm aggregates (Table 3). At 18 hours, the differences between the populations were still present albeit less pronounced (54 versus 33%).

Total soil bacteria detected with acridine orange

The response of the acridine orange detectable bacteria was measured in the same soil preparations used for following serotype AR18. Similar population sizes of acridine orange detectable bacteria were measured in moist and air dried whole soil samples (Table 4). Low percentages of cells elongated in preparations from moist (7.5%) and air dried (6.2%) whole soil, indicating that 30 $\mu g\ ml^{-1}$ of Nal was toxic to the cells. After 18 hours incubation, the density of elongated cells from whole moist soil was estimated to be 1.0×10^7 cells g^{-1} soil which is approximately eleven times greater than the *R. leguminosarum* bv. trifolii serotype AR18 population size of elongated cells detected by immunofluorescence. Although *R. leguminosarum* bv. trifolii serotype AR18 cells are surely present among the acridine orange elongated cells, other morphotypes were detected as illustrated in Fig. 5. The responsiveness of the bacteria to this yeast extract-Nal combination differed among the aggregate size classes (Table 5). For example, some bacteria in the <0.25 mm aggregates had elongated after 6 hours, while those from the 2-5 mm size class did not exhibit a noticeable response until 12 hours. At 18 hours,

Table 4. Population densities of total soil bacteria detected during an 18-hour incubation with yeast extract (0.4 g L^{-1}) and nalidixic acid (30 mg L^{-1}).

Soil samples	No. of cells g^{-1} soil x 10^{-6} after incubation for (h)			
	0	6	12	18
Whole moist soil[‡]	90.0 (± 13.0)	96.8 (± 5.3)	123.0 (± 25.4)	139.0 (± 14.0)
Whole dried soil[1]	67.0 (± 16.7)	67.0 (± 4.2)	92.4 (± 9.8)	97.0 (± 12.9)
<0.25 mm aggregates[2]	77.1 (± 5.4)	72.3 (± 4.3)	68.8 (± 8.2)	82.1 (± 8.2)
2-5 mm aggregates[2]	66.7 (± 9.0)	67.0 (± 12.0)	62.9 (± 9.5)	89.8 (± 15.0)
<0.25 mm aggregates[3]	110.0 (± 6.8)	94.0 (± 15.7)	91.2 (± 17.5)	89.9 (± 12.3)
2-5 mm aggregates[3]	92.1 (± 3.3)	83.7 (± 11.1)	92.1 (± 9.7)	93.4 (± 13.1)

[†]Numbers in parentheses represent the standard deviation of the mean of three replicates.
[‡]Whole moist soil contained 21% water.
[1]Soil dried at room temperature, overnight. Whole dried soil contained 3.1% water.
[2]Aggregates prepared from whole soil dried at room temperature, overnight. <0.25 mm aggregates contained 2.6% water and 2-5 mm aggregates contained 3.4% water.
[3]Aggregates prepared from whole soil dried at 4°C, 7 days. <0.25 mm aggregates contained 3.9% water and 2-5 mm aggregates contained 5.7% water.

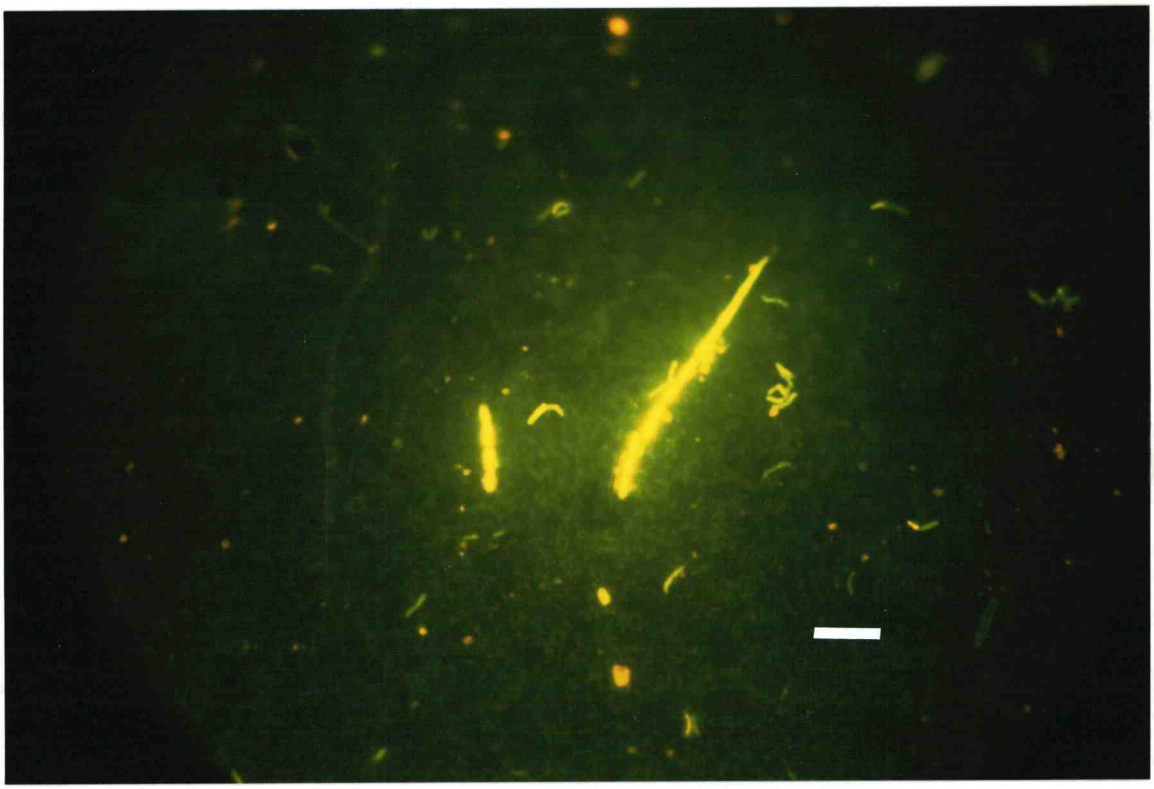

Figure 5. Elongated and morphologically diverse bacteria detected by acridine orange staining after 18 hours incubation. The length of the calibration bar is equal to 10 μm. (Magnified 1000X).

Table 5. Percentage of total elongated soil bacteria detected during an 18-hour incubation.

Soil samples	Time of Incubation (hours) 0	6	12	18
Whole moist soil	0 (± 0)	0 (± 0)	0 (± 0)	7.5 (± 3.1)
Whole dried soil[1]	0 (± 0)	0.1 (± 0.2)	0 (± 0)	6.2 (± 2.2)
<0.25 mm aggregates[2]	0 (± 0)	0.2 (± 0.4)	0.8 (± 0.7)	5.9 (± 3.4)
2-5 mm aggregates[2]	0 (± 0)	0 (± 0)	0.1 (± 0.2)	1.4 (± 1.2)
<0.25 mm aggregates[3]	0 (± 0)	0 (± 0)	0.3 (± 0.4)	13.2 (± 1.4)
2-5 mm aggregates[3]	0 (± 0)	0 (± 0)	0 (± 0)	2.1 (± 0.9)

[†]Numbers in parentheses represent the standard deviation of the mean of three replicates.
[1]Soil dried at room temperature, overnight.
[2]Aggregates prepared from soil dried at room temperature, overnight.
[3]Aggregates prepared from soil dried at 4°C, 7 days.

preparations from the <0.25 mm aggregate fraction contained approximately 6% elongated cell while 2-5 mm aggregates had 1.4% (Table 5).

Aggregates prepared from soil dried at 4°C for 7 days showed similar trends to those exhibited by aggregates prepared by air drying at room temperature overnight. In this case, preparations of the <0.25 mm aggregates contained elongated cells at 12 hours while elongated cells were not detected in the 2-5 mm aggregates until 18 hours (Table 5). After 18 hours incubation, the <0.25 mm fraction had a much higher percentage of elongated cells than did the 2-5 mm fraction (13 versus 2.1%) (Table 5).

DISCUSSION

The detection methods and viability assay used in this study allowed the examination of the antibiotic resistance and sensitivity of soil bacteria, and provided an insight into the different niches occupied by bacteria in soil.

The fluorescent antibody technique was introduced for the detection and enumeration of antigenically distinct soil bacteria many years ago (Schmidt and Bankole, 1962; Bohlool and Schmidt, 1970). While the immunofluorescence technique does not discriminate between live/dead bacteria because the antibody is prepared against antigens of heat killed cells, when combined with a viability assay, bacteria which are viable can be easily detected and distinguished from dead cells. Bottomley and Maggard (1990) combined immunofluorescence with the cell elongation assay of Kogure et al. (1979) to determine the density and viability of serotypes within a soil population. In that study, 10 to 20 $\mu g\ ml^{-1}$ nalidixic acid was sufficient to prevent cell division and promote elongation of several indigenous serotypes of *Rhizobium leguminosarum* bv. trifolii in a pasture soil. Kingsley and Bohlool (1983) used Intrinsic Antibiotic Resistance to several antibiotics, including 10 $\mu g\ ml^{-1}$ nalidixic acid to characterize, group and differentiate among *Rhizobium* isolates from chickpea, *Cicer arietinum*. *R. leguminosarum* bv. phaseoli isolates recovered from root nodules were fingerprinted using their IAR to seven antibiotics, including 5 and 10 $\mu g\ ml^{-1}$ nalidixic acid (Beynon and Josey, 1980).

In my study, a problem arose because *R. leguminosarum* bv. trifolii serotype AR18 exhibited a high level of intrinsic antibiotic resistance (IAR) which had not previously been observed in this biovar of *R. leguminosarum.* Furthermore, while 30 $\mu g\ ml^{-1}$ nalidixic acid inhibited cell multiplication over 12 hours, by 18 hours, immunofluorescence counts had increased indicating perhaps, that a subset of the serotype was even more resistant.

However, the increase in cell numbers might be a consequence of the incubation method. *Rhizobium* are known to exhibit a pleomorphic morphology in the presence of nalidixic acid which is characterized by branching of the otherwise bacillus-shaped bacterium. Both strain and serotype AR18 exhibited this behavior as observed with immunofluorescence between 6-18 hours of incubation and resulted in cells ≥4.2 μm in length. As the elongated cells are exposed to 18 hours of shaking at 250 rotations per minute, the cells might have simply broken apart at the already weakened portions of the cell wall, resulting in fragments which might have been counted as complete elongated cells. In the past this was unobserved, perhaps because Bottomley and Maggard performed the viability assay in the absence of shaking (Bottomley and Maggard, 1990). A second possibility is that during the cell division of *Rhizobium*, the cell elongates to about 4 to 5 μm before a cell wall is formed between the mother and daughter cell and the cells separate. Whether some dividing cells were misconstrued as elongated cells, is unknown because further time points were not obtained, nor was a size analysis of the bacteria performed.

Research of other *Rhizobium* species, however, indicates that a relatively high level of resistance to nalidixic acid is not uncommon. Segovia et al. and Soberón-Chávez and Nájera have isolated nonsymbiotic *R. leguminosarum* bv. phaseoli from soil using resistance to nalidixic acid as a selection criterion; nonsymbiotic rhizobia were recovered from yeast extract mannitol plates containing nalidixic acid at 100 $\mu g\ ml^{-1}$ and 80 $\mu g\ ml^{-1}$, respectively (Segovia et al., 1991; Soberón-Chávez and Nájera, 1989).

In my study, 30 $\mu g\ ml^{-1}$ nalidixic acid was toxic to the majority of soil bacteria as indicated by the low percentages of elongated cells observed after 18 hours of incubation. Interestingly, however, from an ecological standpoint, the method inadvertently allowed me to observe the response of a sub-population of the soil bacteria which was resistant to 30 $\mu g\ ml^{-1}$ Nal and which showed some differences among aggregate size classes. At nalidixic acid concentrations of 20 $\mu g\ ml^{-1}$, upwards of 50% of the total soil bacterial cells were elongated which is similar to the preparation of elongated cells observed by Bottomley and Maggard (1990). Bottomley and Maggard showed that soil extractions incubated with 10 $\mu g\ ml^{-1}$ nalidixic acid and 200 $mg\ L^{-1}$ yeast extract were sufficient to inhibit cell division while allowing approximately 65% of the cells to respond over a 24-hour sampling period (Bottomley and Maggard, 1990). In both cases, the results illustrate that a high proportion of acridine orange stained soil bacteria are responsive to simple nutrients like yeast extract and are viable. Since I did not

make an attempt to enumerate the culturable bacteria within the population, I do not know what proportion of the viable cells exist in a culturable state.

The soil aggregates that microorganisms inhabit are of interest in determining the role that microorganisms play in nutrient availability and the influence of microbial activity on soil physical properties. Researchers have shown that soil microorganisms and their activities can be distributed heterogeneously across all aggregate size classes (Beauchamp and Seech, 1990; Seech and Beauchamp, 1988; Gupta and Germida, 1988; Franzluebbers and Arshad, 1997). In this study, *R. leguminosarum* bv. trifolii serotype AR18 populations were homogeneously distributed among the <0.25 mm and 2-5 mm aggregate size classes and distribution was not influenced by drying. Yet the *R. leguminosarum* bv. trifolii serotype AR18 population present in whole soil fractions was influenced by drying being twice as large as in dried soil. It is possible that populations in aggregate-size classes that I chose not to analyze are more sensitive to soil dessication.

Although the bacteria appear to be uniformly distributed across the aggregate size classes examined, their abilities to respond to substrate and nalidixic acid were different. Elongated cells were visible in the <0.25 mm aggregate and whole dried soil preparations after 6 hours of incubation, while the populations of the 2-5 mm size class lagged and elongated cells were not detected for 12 hours (Table 3 and 5). There are two hypotheses that could account for the differences observed in *R. leguminosarum* bv. trifolii serotype AR18 and total soil bacteria.

First, the different aggregate size classes might offer differential protection from an environmental stress such as soil drying. As a result, the population detected in the 2-5 mm aggregates might be more susceptible to the drying event and therefore composed of a larger number of physiologically compromised bacteria. Second, the *R. leguminosarum* bv. trifolii serotype occupying the 2-5 mm aggregates might be genotypically different from that occupying the <0.25 mm aggregates and express different physiologies and a longer time course might have been required to see a true profile of their response. Further experiments are required to discriminate between these two possibilities.

It is estimated that total soil bacteria population densities typically range between 10^6-10^9 cells per gram of soil and *Rhizobium* populations range from 10^8 cells per gram of soil in the rhizosphere of the host legume to <100 cells per gram of soil in the absence of the host plant (Atlas and Bartha, 1998; Moawad et al., 1984; Bottomley and Maggard, 1990). Mendes reported population densities for serotype AR18 to be approximately 10^6 cells per gram of soil, while the estimates presented here are in the range of 10^4-10^5 cells per gram of soil (Mendes and Bottomley, 1998). Whether this difference in population size is real or is a result of the methods used in detection, is unknown. The N. Willamette field plots have been under investigation since 1989 and it is only recently that microscopic and viability evaluations of the total soil bacteria and *Rhizobium* populations have been initiated and data made available (Mendes and Bottomley, 1998). Further studies are necessary to evaluate the nodulating population of serotype AR18 in order to

determine the size of the symbiotic and nonsymbiotic populations. In screening a soil containing a *R. leguminosarum* population, Segovia et al. found 1 in 40 *Rhizobium* isolates was symbiotic. In comparison, Mendes reported similar results were obtained from immunofluorescence and MPN data from plots planted with the legume (Mendes and Bottomley, 1998).

I believe that the cell elongation assay might also be applied to studies of various aspects of the molecular ecology of soil bacteria. Bacterial populations could be exposed to nalidixic acid and specific substrates and elongated cells captured on 0.45 μm pore-size filters. These recovered cells could be further examined using molecular techniques to characterize the representatives of the soil bacterial populations that responded. The physiological or functional characteristics of the cells could be examined. Conserved genomic sequences could be examined in an attempt to establish a link between its evolutionary place and its ecological role or niche. Bacterial enzymes could be activated through enrichment and again, through capture and use of molecular methods, characterization of the bacterial populations could be done. This information would be useful in further determining the roles of soil bacteria in nutrient cycling, aggregate formation and stabilization, in addition to describing the genetic relatedness of soil bacteria. Inevitably, this would lead to a greater understanding of the species diversity and richness of the soil environment.

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